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METHODS FOR TESTING SHELLFISH FOR POLLUTION.*†

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IN March, 1901, acts were passed by the legislature of Massachusetts, directing the State Board of Health to examine annually the conditions about the main sewer outlets of cities and towns and report thereon, and prohibiting the taking of oysters, clams, quahogs, and scallops from polluted sources. Acting under this authority, investigations were commenced early in that year, the examination of sources and collection of samples being undertaken by the Engineering Department, and the bacteriological analyses being made at the Lawrence Experiment Station. In reporting to the legislature upon this work in January, 1902, the following statements were made: "The outlets along the seashore, by which flats or tidal waters which are sources of shellfish used extensively for food were liable to be polluted, were deemed to be of great importance, on account of the direct injury to health that might result from the use of contaminated food; and the work of the Board during the past year has been directed principally to an examination of the conditions about sewer outlets into the sea and tidal waters." "The analyses have consisted in a determination of the presence or absence of the colon bacillus, the characteristic organism of sewage. In collecting the samples from the various flats and waters in which they occur it has been the custom in most cases to collect with the sample of shellfish a sample of the water upon or near the flats, and analyses have been made both of the water and the shellfish."

At the time these investigations were commenced, little was known about the bacterial flora of shellfish, about the viability of B. coli, the typhoid bacillus, and other organisms in shellfish or in sea-water; in fact it was not definitely known whether the shellfish would take up B. coli, and it was necessary not only to devise satisfactory methods of analyses, but also to conduct experiments to determine how the

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results of analyses should be interpreted. The analytical methods now in use have been developed gradually as the investigations have continued, and while based largely upon results obtained at Lawrence, parts of the methods in use in other laboratories have been adopted or modified when such procedure seemed an improvement. The interpretation of results also, while based upon the Lawrence experiments, have included the many excellent researches conducted both in this country and abroad. In view of the growing importance of the subject and in response to frequent requests for information concerning the methods in vogue in Massachusetts, it is the purpose of the writer in the present article to describe in detail the present practice in the collection and analysis of shellfish samples, and the interpretation now placed upon the results of those methods, and to supplement these remarks by notes as to certain modifications which suggest themselves. As in some instances these notes may be taken as criticisms of the really excellent work of other investigators, all references except to Massachusetts work have been purposely omitted.[1]

As stated previously, complete bacteriological analyses are also made of the samples of sea-water from the same sources as the shellfish samples, the methods of sea-water analysis being practically identical with those for waters of other types. While the water analyses have been of much value, together with the shellfish examinations, in determining the amount of pollution of the various sources examined, it would be unwise to depend upon sea-water analyses alone, since the character of such samples in the majority of cases is influenced by tidal flow, wind, currents, etc. On the other hand, the analyses of the shellfish themselves as made at the present time show the amount of pollution at the time they are gathered and the condition in which they would be marketed and consumed.

COLLECTION OF SAMPLES.

Samples are collected in sterile springtop glass preserve jars holding one pint.[2] In the earlier work, when sampling at growing grounds, a sterile spoon was used to pick up clams and place them in jars. This seems to be an unnecessary refinement, and in recent years the collector has used his fingers, and so far as could be determined the error or chance of contamination by so doing has been negligible. If sterile handling is considered necessary, the individual clams might be picked up with crucible tongs which could be sterilized by the alcohol method before use, or could be kept in a bottle of some sterilizing agent when not in use. The sample from each location should consist of about 15 individual clams, as some may be lost in transportation or during the analysis.

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[1] A complete review of the literature concerning shellfish pollution investigations is given in a report made to the legislature in 1902, published as *Massachusetts Senate Document*, No. 336, 1902.

[2] For oysters, the openings in jars may be so small that only the smaller oysters can be introduced. As only a small proportion of shellfish samples in Massachusetts have been oysters, the use of the glass jars has been continued, small oysters being selected when it is necessary to collect them. Metal boxes, such as lunch boxes, might be used when large oysters must be collected. The collapsible kind could

TRANSPORTATION OF SAMPLES.

It is desirable that samples reach the laboratory as soon after collection as possible. In the earlier work in Massachusetts samples were packed in ice during transit.[1] Experiments upon the viability of B. coli and B. typhosus in clams and oysters, showed that if the weather was not too warm shipment without ice did not affect the analytical results, and in recent years icing has been omitted on shellfish samples which are to reach the laboratory within 24 hours after collection. Possibly two or three days might elapse without seriously affecting the results, but the shorter period is preferable.

INSPECTION OF SAMPLES.

When samples are received at the laboratory they should be inspected, to see if in good condition. If in good condition the shells will be closed tightly or will close tightly as soon as the individual clam or oyster is touched. Individuals with shells open, or which do not close quickly on being touched are either dead or nearly so, and should be rejected.

TECHNIC OF TESTING.

Ten clams or oysters from each location are tested for the presence of B. coli in the shellwater and in the alimentary canal.[2] The

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be conveniently sterilized and if wrapped in paper could be easily carried around or stored until needed. Perhaps a waxed cardboard box would serve the purpose as well, in which case new sample boxes could be used each time. The advantage would be, less weight and the elimination of cleaning and resterilizing.

[1] The shipping case used for shellfish samples packed in ice is similar in construction to the case used for water samples described by the writer, *Technology Quarterly*, December, 1908, p. 516.

[2] Investigations of the relative occurrence of B. coli in different portions of a large number of polluted clams and oysters showed that the test organism could be isolated from the shellwater with the largest percentage of accuracy, and with slightly less accuracy from the intestines (*Report Mass. State*

required number of fermentation tubes should be marked distinctly by some such method as that described later under "Recording" before starting. The technic of testing is as follows: The individual is removed from the sample jar with sterile crucible tongs, and washed with a stream of sterile water.[1] This sterile water should preferably be as hot as can be conveniently handled.[2] The clam being laid upon the left hand and held firmly, the fingers gripping the hinge end, the point of a sterile oyster knife is inserted between the shells and the muscle cut.[3] With a little experience, this may be done without injuring the body of the clam or oyster. The upper shell being removed, a portion of the liquor in the lower shell is now transferred to a fermentation tube with a sterile pipette, or a portion of this shellwater may be carefully poured directly from the shell into the tube. The latter method is much simpler than the use of pipettes, but requires that the shell be so handled in the previous operation that

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Board of Health, 1905, pp. 430 ff.). Since in many methods of cooking, the shellwater is discarded, and on the other hand the shellwater or broth as it is often called is used alone in many instances, it was believed that valuable information would result from separate tests of both shellwater and intestines. The object in testing ten individuals separately is to permit the results of tests to be expressed quantitatively, thus showing to a considerable extent the degree of pollution. Determinations of the numbers of bacteria in the shellwater have been made from time to time but the numbers so determined apparently did not have sufficient diagnostic value to repay the extra labor involved in making them. During the past few years, however, counts of the total and red colonies on litmus lactose agar after 18 hours at 40° C. have been made a part of the routine analyses of samples of water and sewage and the results so obtained have proved a valuable addition to the usual determination of total bacteria at room temperature and qualitative tests for B. coli. No opportunity has been afforded to apply this method to analyses of shellwater, altho some similar counts of sea-water have been made. It is believed, however, that differential counts of this character might prove extremely valuable after their significance had been worked out, and it is quite possible that such counts might entirely supplant the more tedious and less accurate qualitative tests for B. coli as now applied.

[1] A wash bottle for sterile water designed especially for use in shellfish analyses was described by the writer, *Technology Quarterly*, December, 1908, p. 519.

[2] If preferred, the shellfish may be dried with a towel or with a clean sheet of filter paper, dipped in alcohol, and burnt off as described under "Sterilization of Instruments," after the shell has been washed, and the same procedure may be followed when the body has been removed from the shell and washed preparatory to dissection. This seems to be an unnecessary refinement, however, if the samples are carefully handled.

[3] In attempting to open oysters, the beginner would better have a heavy leather or cloth pad to protect the hand from injury in case the knife should slip. This pad may be covered by a sheet of clean waterproof paper to prevent carrying contamination from one sample to another if desired. With a little experience and careful handling this refinement is not necessary, as the pad or hand should never come in contact with the portion of shellfish which is used for inoculating the tubes. The difficulty of opening oysters has led some operators to kill the oyster by immersing in boiling water or laying on a hot plate. Such a process is objectionable on theoretical grounds as the test organisms may be killed also. If care is taken to check the application of heat immediately the shells start to open it is possible the harm done may not be serious and such a process might be allowed in urgent cases. It cannot be recommended. Another method practiced in some laboratories is to bore through the shells with a sterile drill. There is no objection to this if a mash sample of body and shellwater is desired. The separate testing of shellwater and intestine is of course impossible by this process.

the lip over which the liquor is poured has not been contaminated. The body is now washed with sterile water,[1] then while held with the fingers of the left hand, an incision is made with a sterile scalpel and a portion of the intestine transferred with sterile forceps to another fermentation tube,[2] care being taken not to touch the parts where the incision is made with the fingers or to contaminate it in any way. This procedure is repeated until 10 individuals have been tested from each sample jar.[3]

CULTURE METHODS.

The tests for B. coli are made by the well known Lawrence method in exactly the same manner as are similar tests for B. coli in water. Preliminary fermentations in dextrose peptone solution are incubated 18 hours at 40° C. Tubes showing fermentation are plated out on litmus lactose agar, and incubated 18 to 24 hours at 40° C. If red colon-like colonies develop, two or more are fished from each litmus lactose agar plate to agar streaks. If colonies of the sewage streptococcus type are abundant on the plates, or if agar streak is of the streptococcus type, this should be noted also.[4] If agar streaks show characteristics of B. coli after 18 to 24 hours at 40° C., transfers are

[1] See footnote 2, p. 81.

[2] In some laboratories it is the practice to transfer the shellwater and body of the shellfish to a sterile petri dish, and after mashing them up together to inoculate a single fermentation tube with the mixture. Experiments at Lawrence have shown that it is quicker and neater to perform the operations as described above, and if it is necessary to reduce the number of tests to be carried out to inoculate both the shellwater and intestine from each individual into the same fermentation tube. Mixing the contents of the two fermentation tubes after incubation is also practiced in some places, but while this process yields somewhat more information as to the presence of fermenting organisms than does the combined method, experience has shown that the chances of isolating B. coli from such a mixture are considerably reduced, and this is the ultimate aim of the tests. When separate tests of shellwater and intestines can be made, the additional information obtained will amply compensate for the extra labor.

[3] In some instances when a large number of samples was collected on the same day, or when the number of individuals in each jar was not enough, five individuals or even less have been tested. This reduces the quantitative accuracy of the results, and impairs the judgment of the extent of pollution. If the number of tests which can be made is limited by insufficient apparatus or by pressure of other work, combined inoculation of the same tube with both shellwater and intestine is preferable to cutting down the number of individuals tested.

[4] It has been the experience at Lawrence that the streptococcus type is present more frequently in the shellwater of clams, etc., from polluted sources, and in polluted sea-water than in any other class of samples, and as it quite commonly overgrows the colon and other fermenting types, the isolation of such types is at times almost impossible. While B. coli is considered a better index of pollution than is the sewage streptococcus, and while no direct search is made for the latter, the presence of the streptococcus type undoubtedly does indicate pollution and when observed on the litmus lactose agar plates or upon the agar streak it should be recorded. The appearance of the streptococcus on the plate or agar streak is so different from any other types occurring under the conditions imposed by the methods, that it is readily recognized and its confirmation by microscopical examination is entirely unnecessary. See *Report Mass. State Board of Health*, 1901, p. 406.

made to tubes of Dunham's solution, nitrate-peptone solution, dextrose-peptone solution, and gelatin.

Dextrose-peptone and nitrate tubes are incubated 18 to 24 hours at 40° C. after which they are tested respectively for presence of gas or for nitrites. The tubes of Dunham's solution are tested for indol after three days at 40° C. The gelatin tubes are incubated at 20° C. for 14 days, being inspected for liquefaction on the fourth, seventh, tenth, and fourteenth day.

STERILIZATION OF INSTRUMENTS.

The simplest and easiest method of sterilizing the tongs, knives, forceps, etc., used in shellfish analysis is by dipping them into wood or denatured alcohol and then passing them through the flame, allowing the alcohol to burn off naturally. This will effectually destroy small particles of infected material without drawing the temper of the steel. It goes without saying that the instruments should be clean before sterilizing by this process, as any large particles of flesh, etc., would only be sterilized upon the outside. It is better to use instruments composed entirely of metal as the alcohol is apt to soak into the seats of wooden or bone handles, which become charred after being burnt off a few times, allowing the steel blades to become loose.

MEDIA.

The media employed correspond essentially with those recommended by the Committee on Standard Methods of Water Analysis of the American Public Health Association as modified in 1906 and 1907. The principal difference consists in the use of a solution of one per cent dextrose and one per cent peptone (Witte) in tap water in place of the usual Smith's solution, so called, in the fermentation tubes. This medium, as noted by the writer elsewhere,[1] yields satisfactory qualitative fermentation tests, but as the amount of gas formed is much smaller than in media containing beef broth, measurements of gas ratio if they are desired are not as conveniently made. Its chief advantage lies in the greater ease of preparation and in the facts that its composition is more uniform than that of media containing meat, that no adjustment of the reaction is necessary, and that no change of reaction occurs during sterilization.

[1] *Report Mass. State Board of Health*, 1901, p. 399.

Another variation is in the agar media used for streak cultures which contains only one per cent of agar instead of one and one-half per cent as originally recommended by the Standard Methods Committee.

RECORDING AND REPORTING.

When collecting samples of shellfish from the gathering grounds it is better to indicate the sampling stations by letters or numbers, and record the location marks in a notebook together with the station number where they may be available for future reference. The Massachusetts practice has usually been to indicate the stations from which shellfish samples were collected by numbers, and stations from which water samples were collected by letters, locating these stations upon a map or chart which is filed with the analyses and a copy of which is included with the report.[1] On arriving at the laboratory, the jars of samples and the accompanying certificates showing their source are numbered serially. The last one or two figures of the sample number or the location number are marked on the fermentation tubes with a wax pencil when inoculating from the shellfish. In addition, the tubes for each sample are numbered serially from zero to nine, and the tubes inoculated with shellwater and intestine are marked *a* and *b* respectively to enable a complete record to be made of any peculiar characteristics of each individual. These marks may be carried throughout the confirmatory tests if desired. At Lawrence, where an exact record is kept of the detailed behavior of every culture submitted to confirmatory B. coli tests, it has been found more convenient to have a series of culture numbers and to substitute these numbers for the sample marks when agar streak cultures are fished, the sample marks being entered in the B. coli culture book against the culture numbers.

In reporting, the results of shellfish analyses are expressed as percentages of the whole number of individuals tested from each sample, the expression of percentages being facilitated by the fact that ten individuals were tested in each case. The report shows:

I. Percentage of individuals from each sample which ferment dextrose.
    a) shellwater.
    b) intestine.
    ab) both shellwater and intestine.

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[1] Examples of the use of maps upon which the location of sampling stations are marked are to be found in the "Report on Pollution of Boston Harbor," *Report Mass. State Board of Health*, 1905, pp. 413–26.

II. Percentage of individuals from each sample in which B. coli was found present.
  a) shellwater.
  b) intestine
  ab) both shellwater and intestine.

III. Percentage of individuals from each sample in which sewage streptococcus was noted.
  a) shellwater.
  b) intestine.
  ab) both shellwater and intestine.

INTERPRETATION OF RESULTS.

Experiments at Lawrence and elsewhere have shown that the relative viability of B. typhosus and B. coli in clams and oysters and in sea-water, as well as under a variety of other conditions, is very similar and that the presence of B. coli may reasonably be considered to represent the possible presence of B. typhosus. Furthermore, the experiments have shown that these organisms may live in shellfish kept under market conditions for many days, and that the methods of cooking ordinarily employed frequently do not kill these organisms when they are present inside the shellfish.[1] In addition there have been a number of well authenticated epidemics of typhoid fever traced to the eating of polluted shellfish. On the other hand, the vital statistics of cities and towns along the seacoast, where shellfish are plentiful and are consumed in considerable quantities, do not show any marked increase in typhoid fever, other conditions being equal, over places in the interior where the consumption of shellfish is relatively small. And this is in spite of the fact that in some instances the shellfish consumed locally are taken from sources that are grievously polluted.

The investigations at Lawrence showed that B. coli and the sewage streptococcus are absent, or are present in only a small proportion of samples collected from sources which are remote from pollution. Sources which are absolutely free from all suspicion of chance pollution are rare, however, and the test organisms may be present in a small proportion of samples which would nevertheless ordinarily be considered entirely safe. On the other hand, the test organisms are always present in a majority of the samples from sources whose pollution is evident. Even the most seriously polluted sources, however, may yield a certain percentage of samples in which B. coli are not present, or at least in which they cannot be demonstrated. For example, B. coli could not be found in a considerable percentage of samples of clams collected close to the outlet of a large city sewer, altho present in 80 to 90 per cent of samples from the same area at a distance of one-half to one and one-half miles from the sewer outlet.[1]

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[1] "Experiments on Relative Viability of B. coli and B. typhosus," *Report Mass. State Board of Health*, 1902, pp. 268–77. Experiments on "Length of Life of B. coli in Clams, Oysters, and Sea-Water and on Efficiency of Various Methods of Cooking Shellfish," *Report Mass. State Board of Health*, 1905, pp. 429–56.

It should be stated in the strongest terms, that the determination of the presence or absence of B. coli in a single clam or oyster from a given source is of small diagnostic value if we may draw conclusions from the results of many thousand tests made at Lawrence during the last eight years. With a sufficient number of samples, the absence of B. coli, or of positive fermentations followed by an overgrowth of streptococcus, can safely be taken to indicate freedom from pollution, and when 10 individuals are tested, a negative test in 8 out of 10, or 80 per cent of the samples, can be assumed to be indicative of reasonable safety. Under the same conditions, a positive test in 50 per cent or more of the samples must indicate pollution of a more or less dangerous character. Between these extremes, and it is within this limit that a large proportion of sources will be found to fall, the interpretation is a question of individual judgment for which no hard and fast rules can be made. In such cases the sanitary inspection of the source must play an important part, and the extent and condition of the shellfish industry in that particular place, and whether the shellfish are to be eaten raw or are to be cooked before being consumed must also bear some weight if justice is to be accorded to both consumer and producer.[2]

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[1] *Report Mass. State Board of Health*, 1902, pp. 260–62.

[2] Shellfish, such as clams, oysters, quahogs, scallops, and mussels, form an important part in the food supply, and the gathering of these shellfish is an industry in which many persons are employed. In Massachusetts, the value of the shellfish gathered along the shores amounts to about $500,000 per year or about one-tenth of the entire income from the fisheries, about one-half of this being from oysters, about one-fourth from clams and quahogs, and the remainder from scallops, mussels, etc. Altho the oyster catch is the most valuable, the great bulk of the food supply from this source is composed of clams and quahogs, of which some 400,000 bushels are gathered annually, while the oyster catch is only about 55,000 bushels, and the scallop catch about 90,000 gallons. About 90 per cent of the shellfish consumed in Massachusetts are produced within the state. Relatively a small proportion of the clams consumed are imported, while perhaps half of those gathered within the state are exported to other states. On the other hand, probably more than half the oysters consumed are brought in from outside, while only a small proportion of those gathered within the state are exported. For this reason the control of the pollution of the shellfish gathering grounds within the state is of the utmost importance as affecting the health of the community. As most of the gathering grounds are the property of the state or of the cities and towns, and as access to these is free to all, the investment required to engage in these industries is small while the returns on that investment are quite large. The industry is not organized and is composed largely of individuals who work separately or in small groups and who in the majority of cases market their catches themselves.